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The analysis of peptide affinity and its binding kinetics to DR1DW1 major histocompatibility complex protein

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Abstract

The connection between experimentally measured values of ED_{50} (concentration of added peptide required to bind half of the protein), which characterize peptide–protein binding and the equilibrium dissociation constant of peptide–protein complex K_d (affinity) is considered. It is shown and confirmed by experimental studies that in certain cases, as a result of the absence of equilibrium in the system, the value of K_d could be much less than the experimental value of ED_{50}, but not equal to that as commonly assumed. This is especially applicable to the formation of peptide–MHC complexes with low dissociation rates (strong binding), which may require longer time-intervals to reach equilibrium. Thus the search of the good binding peptides based on finding ones with the smallest measured values of ED_{50} may result in missing the best binders with the lowest values of dissociation constant (highest affinity). To analyze the problem we considered the formal chemical kinetics of peptide–protein binding. Experimental studies of peptide binding was performed to obtain the parameters of the kinetic model. According to the predictions of the model, it was confirmed that peptide binding occurs through the preceding step, which is either a release of an endogenous peptide or some conformational change of the molecule. The half decay time for this process was determined to be approximately 3 h. Based on the model developed, a new effective method for determination of the dissociation rates of peptide–MHC complexes and the equilibrium dissociation constants K_d was proposed, which implies the comparison of binding levels (ED_{50}) at different instants of time. This method works especially well for the peptide–MHC complexes with relatively slow dissociation rates (stable complexes), for which the direct off-rate measurements as well as obtaining equilibrium binding data to determine K_d are highly time consuming and not very reliable.

Keywords: Peptide–MHC protein binding; Two-step binding kinetics; Peptide–protein affinity and ED_{50}; Association and dissociation rates of peptide–protein binding

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1. Introduction

Peptide binding to class II major histocompatibility complex (MHC) proteins is a central step in immune recognition. MHC class II molecules are found in all vertebrates on special cells of the immune system such as macrophages, dendritic cells, and B-cells. These cells proteolytically degrade foreign protein into peptides, which then bind to the class II molecules [1]. MHC–peptide complexes are transported to the cell surface and engage in further steps of immune response. MHC class II protein are composed of two α (32 kDa) and β (28 kDa) subunits, which are non-covalently associated. The details of MHC class II proteins structure are rather well known [2,3].

Each MHC molecule can bind to a variety of peptides. The MHC–peptide complex is relatively long-lived and dissociates with a half-life of the order of hours or days. The molecular mechanisms by which peptides bind MHC molecules are not well known, however. The interpretation of the kinetics of peptide binding is not straightforward. A molecular mechanism can never be proven in a rigorous sense from kinetic data, but kinetic measurements can prove a mechanism and show that it is consistent with observation. Kinetic mechanism frequently leads to predictions that are capable of experimental test, that is why kinetic data furnish the most direct clues for discovery of the mechanism of the process.

The binding of peptides to MHC molecules was studied in several articles [3,4,13,14]. For the purpose of peptide-based drug development it is usually important to find peptides with high affinity to the MHC proteins. The evaluation of peptide affinity based on the experimental measurements of ED_{50} (the concentration at which binding curve reaches half of the plateau value) may in certain situations be misleading. As a result the best binding peptides (with smallest values of K_d, or highest affinity) will be missed if the experimental studies are not conceived properly. Numerical simulations for peptide–MHC binding [14] showed that the value of dissociation constant derived from the Scatchard plot of one of this simulations is similar to the experimentally reported value, but is of three orders of magnitude greater than K_d used in the simulations, thus indicating that the apparent K_d values derived from Scatchard plots (which are approximately the same as ED_{50} values) have no clear significance.

We developed the method for obtaining the dissociation rate of complexes and the equilibrium constant of binding reaction K_d (which appears to be the true affinity of peptide to protein) from the experimentally measured values of ED_{50} at different time points. This method does not require the system to reach equilibrium and thus appears to be highly time saving.

In the present work we have studied peptide binding with human MHC class II molecules DR1DW1 (the abbreviation DR is often used hereafter) in detergent solution. We systematically considered the certain kinetic model of binding, obtained parameters of this model, and studied the limiting cases of the process. In Section 2 we analytically solved the model to predict some experimental results to confirm the validity of this model and also to develop further (Section 5) a novel method for obtaining the dissociation rate (off-rate) constants and equilibrium dissociation constants of peptide–MHC complexes. Experimental procedures for the observation of peptide binding to MHC molecules are described in Section 3. In Section 4 we discuss experimental results and obtain the parameters of the peptide–protein binding model.

2. Formulation and analysis of the problem

The widely assumed mechanism of MHC–peptide complex formation is a two-step reaction [3]

$$\mathrm{DR} \overset{k_e}{\Rightarrow} \mathrm{DR}_{\mathrm{open}} \tag{1}$$

$$\mathrm{DR}_{\mathrm{open}} + \mathrm{P} \underset{k_2}{\overset{k_1}{\Leftrightarrow}} \mathrm{DR{-}P} \tag{2}$$

with a possible irreversible decay of DR

$$\mathrm{DR}_{\mathrm{open}} \overset{k_3}{\Rightarrow} \alpha + \beta \tag{3}$$

Reaction (1) means that either the lost of endogenous peptide takes place [5] or some conformational change of MHC molecule occurs. DR_{open} indicates some intermediate state of DR molecule. We do not take into account the reverse process at Reaction (1), because in all experimental studies the DR concentration was much lower than that of peptide, so that DR_{open} either binds to peptide or dissociates through Reaction (3). The more detailed discussion on reversibility of Reaction (1) is given in Section 4.

The time evolution of Reactions (1)–(3) from the point of view of formal chemical kinetics is described by the system of differential equations. Using the following notations for concentrations $[DR] = D$, $[P] = P$, $[DR{-}P] = C$ and $[DR_{open}] = Q$, we have

$$\begin{aligned} \dot{D} &= -k_e D \\ \dot{Q} &= k_e D + k_2 C - k_1 P Q - k_3 Q \\ \dot{C} &= k_1 P Q - k_2 C \\ \dot{\alpha} &= \dot{\beta} = k_3 Q, \end{aligned} \tag{4}$$

where a superimposed dot denotes ordinary differentiation with respect to time t. The initial conditions for Eqs. (4), which correspond to experimental studies are

$$D = D_o,\ P = P_o,\ C = 0 \text{ and } Q = 0 \text{ at } t = 0. \tag{5}$$

We consider the concentration of peptide to be constant in Eqs. (4), $P = P_o = \text{const}$. This is a reasonable assumption because in all experiments performed the concentration of peptide was much greater than the concentration of DR. The system of Eqs. (4) is then linear and can be solved analytically

$$D(t) = D_o \exp(-k_e t) \tag{6}$$

$$\begin{aligned} C(t) = D_o k_e k_1 \Bigg\{ & \frac{\exp(-k_e t)}{(\lambda_1 - k_e)(\lambda_2 - k_e)} \\ & + \frac{\exp(-\lambda_1 t)}{(k_e - \lambda_1)(\lambda_2 - \lambda_1)} \\ & + \frac{\exp(-\lambda_2 t)}{(k_e - \lambda_2)(\lambda_1 - \lambda_2)} \Bigg\} \end{aligned} \tag{7}$$

$$\begin{aligned} Q(t) = D_o k_e \Bigg\{ & \frac{(k_2 - k_e)\exp(-k_e t)}{(\lambda_1 - k_e)(\lambda_2 - k_e)} \\ & + \frac{(k_2 - \lambda_1)\exp(-\lambda_1 t)}{(k_e - \lambda_1)(\lambda_2 - \lambda_1)} \\ & + \frac{(k_2 - \lambda_2)\exp(-\lambda_2 t)}{(k_e - \lambda_2)(\lambda_1 - \lambda_2)} \Bigg\} \end{aligned}$$

$$\begin{aligned} \alpha(t) = \beta(t) = D_o k_e k_3 \Bigg\{ & \frac{(k_2 - k_e)(1 - \exp(-k_e t))}{k_e(\lambda_1 - k_e)(\lambda_2 - k_e)} \\ & + \frac{(k_2 - \lambda_1)(1 - \exp(-\lambda_1 t))}{\lambda_1(k_e - \lambda_1)(\lambda_2 - \lambda_1)} \\ & + \frac{(k_2 - \lambda_2)(1 - \exp(-\lambda_2 t))}{\lambda_2(k_e - \lambda_2)(\lambda_1 - \lambda_2)} \Bigg\}, \end{aligned}$$

where

$$\begin{aligned} \lambda_{1.2} = \Big\{ & k_1 P + k_2 + k_3 \\ & \pm \left[(k_1 P + k_2 + k_3)^2 - 4 k_2 k_3 \right]^{1/2} \Big\} / 2. \end{aligned} \tag{8}$$

Several important consequences follow from the kinetics of the system (7). If irreversible step (3) exists, $k_3 \neq 0$, then concentration of complexes should go to zero at a long time limit [bell-shape curve for $C(t)$ as a function of time]. If step (1) exists, then the dependence of complex concentration should have a zero derivative at initial instant of time, i.e. $dC/dt = 0$ at $t = 0$. This was observed in the experimental studies [4] (Fig. 1a,c of the article), though the authors did not give any interpretation of the fact.

When the concentration of peptide is relatively high, so that $k_1 P + k_2 \gg k_e$, and the decay of DR does not take place, Reaction (1) becomes the limiting step of binding and the solution of the system (7) simplifies

$$C(t) = C_{eq}[1 - \exp(-k_e t)] \tag{9}$$

$$\begin{aligned} C_{eq} &= D_o k_1 P / (k_1 P + k_2) \\ &= D_o / (1 + K_d / P), \quad K_d = k_2 / k_1. \end{aligned} \tag{10}$$

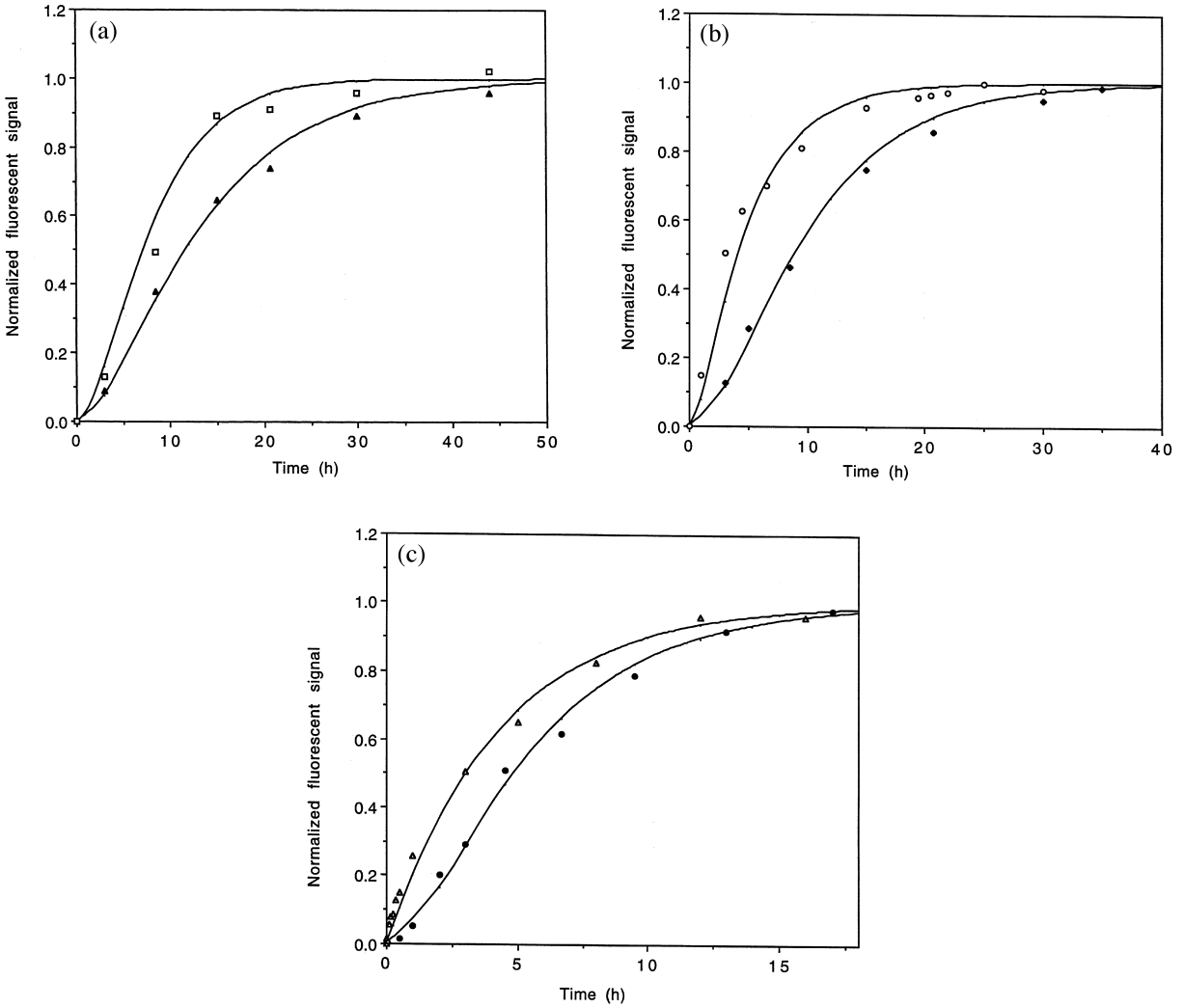

Fig. 1. Kinetics of peptide–DR complexes formation for different initial peptide concentrations. Panel A: ▲, 50 nM; and □, 120 nM. Panel B: ♦, 80 nM; and ○, 500 nM. Panel C: ●, 300 nM; and △, 20 μM. Solid lines are calculated by Eq. (13) with $k_e = 0.23$ h^{-1}, $k_1 = 2$ μM^{-1} h^{-1} and $k_2 = 0$. Each point represents the mean of quadruplicate.

In this case the dimensionless kinetic curve $C(t)/C_{eq}$ does not depend on peptide concentration and is characterized by only a single parameter k_e, which is the rate constant of the preceding Reaction (1).

For relatively low concentrations of peptide, when $k_1P + k_2 \ll k_e$ (but still $P \gg D_o$), and DR does not decay, we get the following equation for complex formation

$$C(t) = C_{eq}\{1 - \exp[-(k_1P + k_2)t]\} \quad (11)$$

or

$$\ln\{C_{eq}/[C_{eq} - C(t)]\} = (k_1P + k_2)t,$$

which formally corresponds to the reaction of direct binding

$$\mathrm{DR} + \mathrm{P} \underset{k_2}{\overset{k_1}{\Leftrightarrow}} \mathrm{DR{-}P} \quad (12)$$

without preceding step (1). In this case the time

scale of Reaction (1) is much shorter than that of Reaction (2), which effectively leads to a simple one-step binding kinetics [Eq. (12)].

At low peptide concentrations a peculiar phenomenon of decreasing of peptide–MHC binding rate with the increase of added peptide concentration (the so-called 'negative' $t_{1/2}$ plots) was observed [3,13]. This interesting problem is considered in a separate publication [15].

Theoretical calculations of reaction rate constants [7–10] are very complicated and require rather detailed information about the system, which is often not available. In this work we used experimental data to test the validity of the model (1)–(3) and to obtain the reaction rate constants.

3. Experimental part

We made binding experiments for 50 nM, 80 nM, 120 nM, 300 nM, 500 nM, 800 nM, 5 μM and 20 μM concentrations of peptide with 3 nM concentration of DR to compare the results with model predictions and to obtain the model parameters. It is known that only some part of MHC molecules, probably because of irreversibly bound endogenous peptides, is reacting with the added peptide to form complexes [1,5,14]. Approximately 10–15% of the DR1DW1 molecules used in this studies were reactive. Thus effectively the concentration of the protein in the experiments was approximately 0.3–0.45 nM.

3.1. Materials and methods

3.1.1. MHC class II protein purification

DR1DW1 molecules were purified from the EBV (Epstein–Barr virus) transformed B cell line by immunoaffinity chromatography as described in detail in the article [11]. It is known that MHC protein which does not have an endogenous peptide in its binding grove is unstable and falls apart [16]. It can be stabilized by adding the peptide, as it was actually done in our studies. That is why the initial zero concentration of DR_{open} in Eqs. (1)–(3) was assumed and the experimental data confirmed the validity of such consideration.

3.1.2. Peptide synthesis

Peptides were synthesized using solid phase techniques [6] either using an Applied Biosystems Peptide synthesizer or an Advanced Chemtech robotics system with commercially available Wang resins, and Fmoc-protected amino acids. Peptides were biotinylated on the amino terminus by treatment on the resin with long chain biotin (LCB) in the presence of HBTU/Hobt dissolved in *N*-methyl pyrrolidinone. The peptides were cleaved from the resin and the side-chain protecting groups were removed simultaneously by treatment with 97% trifluoroacetic acid (TFA) and 3% dodecanethiol for 2 h at room temperature. The TFA was removed by evaporation and the side chain protecting groups were extracted with ethyl ether. The peptides were dissolved in 10% acetic acid, separated from the resin by filtration and lyophilized. The crude peptides were purified by HPLC using a water–acetonitrile gradient with 0.08% TFA in both buffers. The structures of peptides were confirmed by amino acid analysis and mass spectroscopy methods. Peptide used for binding experiment was HA 307–319 (PKYVKQNTLKLAT), which is known to form relatively stable complexes with a half-life of the order of 100 h.

3.1.3. Binding experiments

DR1DW1–peptide complexes were formed at 37°C by incubating 3 nM of DR1DW1 with biotinylated peptide in phosphate buffer saline (PBS; Cellgro, Mediatech, Herndon, VA) supplemented with 0.1 M potassium phosphate, brought to pH 6.5 with NaOH and containing 1% of octylglucoside (OG; Calbiochem, La Jolla, CA). Reaction aliquots were removed at the certain time points and the association reaction was terminated by transferring the aliquots to a 4°C antibody plate. To separate bound from free peptide, complexes were incubated for at least 12 h on a 96-well EIA antibody plate (Costar, Cambridge, MA), which was coated with 5 μg/ml anti-DR1DW1 antibody LB3.1 overnight, blocked with PBS supplemented with 5% fetal calf serum (FCS; Hyclone, Logan, UT) for 1 h at room temperature and then washed with PBS containing 0.02% Tween-20 and 0.05% sodium azide (both from Sigma, St. Louis, MO). The biotinylated peptide–DR complexes were quantified by incubating at 4°C with 100 ng/ml

Europium–streptavidin (Wallac-LKB-Pharmacia, Gaithersburg, MD) in Europium assay buffer [calcium and magnesium free PBS plus 0.5% Bovine Serum Albumin (BSA, Calbiochem), 20 μM diethylenetriamine-pentaacetic acid (DTPA, Sigma) and 0.1% sodium azide] for an overnight period. Washed plates were incubated for 1 h with enhancement solution (Wallac-LKB-Pharmacia), which releases europium from streptavidin and forms a highly fluorescent micellar solution. The resultant fluorescence was measured using a fluorescent plate reader (DELPHIA, LKB-Pharmacia).

4. Results and discussion

Representative binding experiments are shown on Fig. 1. For all binding experiments the plateau was reached with practically the same values of the plateau signal for the same concentrations of DR and no downfall of the curves was observed even for a long time interval of several days. That indicates the insignificance of DR decay, so that we assume $k_3 = 0$ in Eq. (3). Then Eq. (7) for kinetics of the system becomes more simple

$$C(t) = C_{eq}\left[1 + \frac{k_e \exp(-(k_1P + k_2)t)}{-(k_1P + k_2)\exp(-k_e t)} \over (k_1P + k_2 - k_e)\right] \qquad (13)$$

According to experimental data at concentrations of peptide greater than 800 nM the binding curves remain practically unchanged, and independent of peptide concentration. This indicates that there is no 'empty' population of DR and Reaction (1) appears to be the limiting stage of binding, so that binding kinetics for high concentrations of peptide is described by Eq. (9). Thus we obtain the half-life time of the induction reaction (1) $t^e_{1/2} = \ln 2/k_e = 3$ h, which is actually the time interval when binding curves for high concentrations of peptide reach half of the maximum value (plateau), or $k_e = 0.23\ \mathrm{h}^{-1}$. The very close values of the plateau signals mean, according to Eq. (10), that equilibrium dissociation constant $K_\mathrm{d} = k_2/k_1$ is much less than 50 nM. It should be kept in mind that our analysis considers only the region of peptide concentrations $P \geq 10D_o$, which is $P \geq 4.5$ nM for the experiments performed.

Once k_e is known, we can find the value of $k_1P + k_2$ for each binding curve by fitting the equation for $C(t)/C_{eq}$ [Eq. (13)] to experimental points. It turned out that for any different concentrations of peptide P_1 and P_2 the ratio $(k_1P_1 + k_2)/(k_1P_2 + k_2)$ was nearly equal to the ratio of concentrations P_1/P_2. It means that the value of k_2 is much smaller than the value of k_1P and cannot be obtained from the experiments performed. As a convenient approach to this problem, we developed (Section 5) a novel method for obtaining dissociation rate constant k_2 from relatively simple data on binding.

The direct measurement of the off-rate of HA 307–319 peptide through monitoring the decay of DR–peptide complexes gives the half-decay period $t_{1/2}$ approximately 100 h, that corresponds to the value of the off-rate constant $k_2 = \ln 2/t_{1/2} \sim 10^{-2}\ \mathrm{h}^{-1}$. All experimental data fit well by theoretical curve [Eq. (13)] with $k_1 = 2\ \mu\mathrm{M}^{-1}\ \mathrm{h}^{-1}$ and $k_e = 0.23\ \mathrm{h}^{-1}$. The value of k_2 was neglected (assumed zero) in processing experimental data, because in all experiments $k_1\mathrm{P} \gg k_2$. The experimental data for the time of reaching the half maximum (plateau) for binding curves and its numerically calculated values from Eq. (13) are compared on Fig. 2.

The kinetic data obtained give a certain confirmation that Reaction (1) is the lost of an endogenous peptide. Only if Reaction (1) does represent the dissociation of an endogenous peptide, then its reverse direction is suppressed by an excess of added peptide, which binds competing with an endogenous peptide. Though the other possibility is to assume that Reaction (1) is a conformational change of the molecule, the reverse direction of which is suppressed by an excess of binding peptide.

We could not use the derived equations for processing the possible experiments at low concentrations of peptide, when k_1P is of the same order of magnitude as k_2, because it would require the concentration of peptide comparable with concentration of DR. In this case depletion of peptide concentration is significant and should

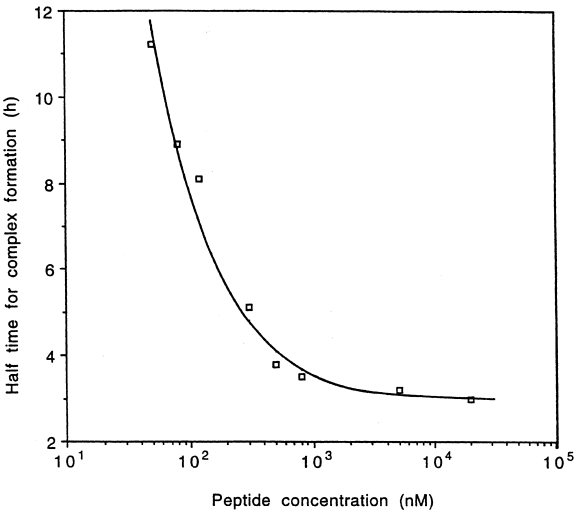

Fig. 2. Half-times for the formation of DR–peptide complexes: □, experimental data; solid line represents analytical calculation using Eq. (13) with $k_e = 0.23\ \mathrm{h}^{-1}$, $k_1 = 2\ \mu\mathrm{M}^{-1}\ \mathrm{h}^{-1}$ and $k_2 = 0$.

be taken into account, so that equations derived earlier are not valid. As shown in Section 2, for the very low concentrations of peptide, when the rate of the preceding step (1) is much faster then the rate of binding, the process can be considered as simple binding [Eq. (12)] with initial conditions $C = 0$, $P = P_o$ and $D = D_o$ at $t = 0$. Kinetic equations for one reaction can be solved analytically in the whole range of initial concentrations P_o

$$C(t) = \frac{\delta_1\delta_2[1 - \exp(-k_1(\delta_1 - \delta_2)t)]}{\delta_1 - \delta_2\exp(-k_1(\delta_1 - \delta_2)t)} \qquad (14)$$

$$P(t) = P_o - C(t)$$

$$D(t) = D_o - C(t)$$

where

$$\delta_{1.2} = \left\{k_2 + k_1(D_o + P_o) \pm \left[\{k_2 + k_1(D_o + P_o)\}^2 - 4k_1^2 D_o P_o\right]^{1/2}\right\}/(2k_1).$$

In Eq. (14) $\delta_2(P_o)$ is the equilibrium concentration of complexes for a simple binding reaction. Really, for equilibrium conditions $(t \to \infty)$ from Eq. (14) we get $C(t \to \infty) = C_{eq} = \delta_2 = \{k_2 + k_1(D_o + P_o) - [\{k_2 + k_1(D_o + P_o)\}^2 - 4k_1^2 D_o P_o]^{1/2}\}/(2k_1)$. It can be easily obtained that $C_{eq}(D_o/2 + K_\mathrm{d}) = C_{eq}(P_o \to \infty)/2 = D_o/2$, so that

$$\mathrm{ED}_{50} = D_o/2 + K_\mathrm{d}. \qquad (15)$$

If $K_\mathrm{d} \gg D_o$ we have $\mathrm{ED}_{50} = K_\mathrm{d}$. Thus the assumption that $\mathrm{ED}_{50} = K_\mathrm{d}$ is valid for the equilibrium of a simple binding reaction only when the concentration of the receptor (protein) used in experiment is much less than the measured value of ED_{50}.

When concentration of peptide is much greater than concentration of DR $(P_o \gg D_o)$, Eq. (14) transforms into Eq. (11) derived under the assumption of an excess of peptide. We did not study binding at low concentrations of peptide to obtain the value of the off-rate constant k_2. Experiments of this kind require long time intervals and also need to invoke the question about how much DR was involved in binding (occupancy level), which is not completely clear. We developed a novel method for obtaining the dissociation rate constant, which is described in the next section.

Thus binding experiments enabled us to find the rate of preceding step (1) k_e and the on-rate of peptide k_1. It is importantly to mention that k_e and k_1 were found consequently: k_e was obtained first from the data at high concentrations of peptide and then association constant k_1 was found from the data at lower peptide concentrations. The advantage of the method presented is that it does not require to use complex non-linear programming algorithms to proceed experimental data to obtain the model parameters, like it was typically done in the other studies [12].

5. The connection between equilibrium dissociation constant (affinity) and experimental data on ED_{50} and a convenient method for obtaining the dissociation rate constant from ED_{50} measurements

In the studies of peptide binding to MHC molecules the so-called binding curves are usually generated. They give the value of fluorescent signal, which is proportional to the concentration

of complexes vs. concentration of the added peptide. The value of peptide concentration at which half magnitude of the curve plateau is reached is called ED_{50} (50% effective dose). Obviously ED_{50} depends on the duration of binding experiment. Usually the values of ED_{50} are viewed as the equilibrium dissociation constants. Really in equilibrium, from Eq. (10) we have $C_{eq}(P=K_d)=D_o/2=C_{eq}(P\to\infty)/2$. The observed value of ED_{50} is equal to the value of K_d only if equilibrium is reached, but this is not valid in general. ED_{50} here does not include the term $D_o/2$ as in Eq. (15), which is neglected when the region of concentrations $P \gg D_o$ is considered (the ratio C_{eq}/D_o depends only on P and K_d, but does not depend on D_o). The analytical approach to the description of the binding model developed in Section 2 enables us to consider the problem of connection between the experimentally measured value of ED_{50} and the value of equilibrium dissociation constant. Using Eq. (13) for obtaining ED_{50} we assume that the value of half maximum of binding curve will always be reached at concentrations at least an order of magnitude greater than MHC concentration, so that the assumption used in Eqs. (7) $P=$ const is satisfied. The plateau concentration, when $P\to\infty$, according to Eq. (13) is

$$C_{max}=C_{max}(t)=D_o[1-\exp(-k_e t)]. \tag{16}$$

The equation for the concentration at which half maximum is reached $P^*=ED_{50}$ is then obtained from Eqs. (13),(16)

$$\left[1+\frac{k_e\exp(-(k_1P^*+k_2)t)-(k_1P^*+k_2)\exp(-k_e t)}{k_1P^*+k_2-k_e}\right]=[1-\exp(-k_e t)]/2, \tag{17}$$

or introducing the dimensionless variables $x=ED_{50}/K_d$, $u=t_{1/2}/t^e_{1/2}$ and $v=t/t_{1/2}$ we rewrite Eq. (17) as

$$\left[1+\frac{u\cdot 2^{-v(1+x)}-(1+x)\cdot 2^{-uv}}{1+x-u}\right]=(1-2^{-uv})/2. \tag{18}$$

As follows from Eq. (18), ED_{50}/K_d depends on two dimensionless variables $t/t_{1/2}$, which is the ratio of reaction (incubation) time to the off-rate time (defined through $t_{1/2}=\ln 2/k_2$) and $t_{1/2}/t^e_{1/2}=k_e/k_2$, which is the ratio of the off-rate constant of the preceding step (1) k_e to the dissociation rate constant k_2 of peptide–DR complexes.

When $t\gg t_{1/2}$ and $t_{1/2}\geq t^e_{1/2}$ we get $x=1$ or $ED_{50}=K_d$. Thus the experimental value of ED_{50} appears to be the value of equilibrium dissociation constant only when reaction time is much greater then the off-rate time scale of binding

Table 1
Ratio of ED_{50}/K_d at 4, 8, and 24 h reaction time as a function of half-life time $t_{1/2}$ of peptide–DR complexes for $t^e_{1/2}=3$ h

$t_{1/2}$ (h)	Reaction time					
	$t=4$ h		$t=8$ h		$t=24$ h	
	$t/t_{1/2}$	ED_{50}/K_d	$t/t_{1/2}$	ED_{50}/K_d	$t/t_{1/2}$	ED_{50}/K_d
1	4	1.288	8	1.077	24	1.0016
10	0.4	5.45	0.8	2.65	2.4	1.16
20	0.2	10.25	0.4	4.75	1.2	1.60
40	0.1	19.75	0.2	8.85	0.6	2.59
80	0.05	38.95	0.1	17.15	0.3	4.63
120	0.033	58.25	0.067	25.45	0.2	6.70
240	0.017	116.25	0.033	50.35	0.1	12.90
360	0.011	173.45	0.022	75.30	0.067	19.10
480	0.008	230.85	0.017	100.55	0.05	25.30

reaction $t_{1/2} = \ln 2/k_2$, while preceding step time scale $t^e_{1/2}$ is of the same order or less than $t_{1/2}$.

Eq. (18) can be solved numerically for different values of the parameters u and v, so that two-dimensional tables for ED_{50}/K_d can be generated. In our case, when $t^e_{1/2} = 3$ h, we calculated the values of ED_{50}/K_d for different possible values of $t_{1/2}$ and for reaction times $t = 4$, 8 and 24 h. The results are given in Table 1. It shows that experimental values of ED_{50} can differ from equilibrium dissociation constant by several orders of magnitude. So that any arguments on changing of ED_{50} in terms of thermodynamics (Gibbs' free energy, entropy, etc.) are not consistent until half-life time of peptide–MHC complex is known and the true value of dissociation constant is obtained, for consideration of which thermodynamic functions are appropriate.

The value of ED_{50} shows how well peptide binds in a certain time interval and includes both equilibrium and kinetic features of binding, that is why the data on ED_{50} only appear to be incomplete information about the system and does not have clear significance in the sense of affinity of peptide to protein. This is especially obvious for peptides with zero off-rates $k_2 = 0$, which equilibrium dissociation constants $K_d = 0$, but non-zero values of ED_{50} will be observed. In this case $ED_{50}(t)$ depends on D_o, k_e and k_1P. The equilibrium limit of ED_{50} for non-dissociating complexes, when irreversible release of an endogenous peptide occur, is $ED_{50}(t \to \infty) = D_o/2$.

In general the value of ED_{50} can never be smaller than half of the concentration of MHC molecules that bind peptide. This lower limit of ED_{50} corresponds to the binding of a peptide with zero off-rate $k_2 = 0$ and reaction time t much longer than $(k_1P)^{-1}$ and k_e^{-1}, so that the system reaches equilibrium. Practically, when reaction time is of order of magnitude greater than $t_{1/2}$ and the half-life time of preceding step (1), the observed value of ED_{50} will be very close to the value of equilibrium dissociation constant K_d (Table 1).

Actually observations of ED_{50} at two different time points appear to be the basis of the novel method which enables us to obtain the off-rate of peptide and the equilibrium dissociation constant. Using Table 1 we can predict the off-rate of peptide based on the ratio of ED_{50} values measured at two different instants of time, for example at 4 and 24 h, or at 8 and 24 h. This is presented in Table 2. It is important that the value of the off-rate $t_{1/2}$ depends only on the ratio of ED_{50}s, but not on their absolute values.

Thus, if the ratio of ED_{50} values is known (column 2 or 3 of Table 2), the value of $t_{1/2}$ can be obtained and then, using Table 1 and the observed values of ED_{50}, we can determine the equilibrium dissociation constant (and then the on-rate constant k_1 as well). This method works especially well for peptides with slow off-rates, when direct observation of peptide–MHC dissociation is a very time consuming experiment, while the ratio of ED_{50} values at different time points increases with the increase of $t_{1/2}$. It is enough to measure ED_{50} at least at two time points. To check the consistency and to control the result obtained, one more time point for ED_{50} can be taken, so that there will be three pairs of ED_{50}, which should give the same values of the off-rate and the equilibrium dissociation constant.

To use this method the measured values of ED_{50} should be at least 10 times greater than the concentration of protein, because in all equations above peptide concentration was assumed to be constant. This condition can be satisfied in the experimental studies either by using lower concentrations of protein or by reducing the incubation time (time of binding reaction) to get higher

Table 2
Ratios of ED_{50} values at 4, 8 and 24 h reaction time as a function of half-life time $t_{1/2}$ of peptide–DR complexes for $t^e_{1/2} = 3$ h

$t_{1/2}$ (h)	ED_{50} (4 h)/ ED_{50} (24 h)	ED_{50} (8 h)/ ED_{50} (24 h)
1	1.286	1.075
10	4.70	2.28
20	6.40	2.97
40	7.62	3.41
80	8.41	3.70
120	8.69	3.80
240	9.01	3.90
360	9.08	3.94
480	9.12	3.97

values of ED_{50}. Thus this method allows us to obtain small values of K_d from non-equilibrium measurements of ED_{50} without determination of the concentration of reacting protein (portion of the protein involved in binding). It is much more convenient than performing a long binding experiment, when equilibrium is reached, so that Eq. (15) is applicable. To use this equation to find K_d, the exact concentration of reacting protein D_o should be known. Then an additional experiment to find D_o would be required.

It is importantly to mention that ED_{50}/K_d increases with the increase of the stability of peptide–protein complex (increase of complex half-life $t_{1/2}$) not as a result of existing of the induction step (1) in the binding mechanism. The same behavior of ED_{50}/K_d takes place for a simple binding reaction [Eq. (12)]: more stable complexes will exhibit greater difference between ED_{50} and the affinity K_d. The equation for the ratio $x = ED_{50}/K_d$ in this case can be easily obtained from Eq. (18) assuming $u \to \infty$ ($t^e_{1/2} \to 0$).

$$(1 - 2^{-\upsilon(1+x)})x/(1+x) = 1/2 \qquad (19)$$

The dependence of ED_{50}/K_d as a function of $\upsilon = t/t_{1/2}$ obtained from this equation is shown on Fig. 3. From Eq. (19) follows that $ED_{50}/K_d \approx (t/t_{1/2})^{-1}$ for small values of $t/t_{1/2} \leq 0.1$.

Using this graph (Fig. 3), tables similar to the Tables 1 and 2 can be generated for a simple binding reaction to determine the half time $t_{1/2}$ of the complex dissociation and the equilibrium dissociation constant K_d from the experimental values of ED_{50} measured at different time points, for example 4, 8 and 24 h. To use this method the measured values of ED_{50} should be at least 10 times greater than the concentration of protein, because in all equations above peptide concentration was assumed to be constant. As before, to satisfy this condition to get higher values of ED_{50}, either lower concentrations of protein or shorter time of binding reaction should be applied. Though for a simple binding reaction we can calculate the ratio ED_{50}/K_d for the whole range of peptide concentrations using analytical description of this reaction (14). In this case ED_{50}/K_d depends on two parameters $t/t_{1/2}$ and D_o/K_d, which makes analysis of experimental data more complicated.

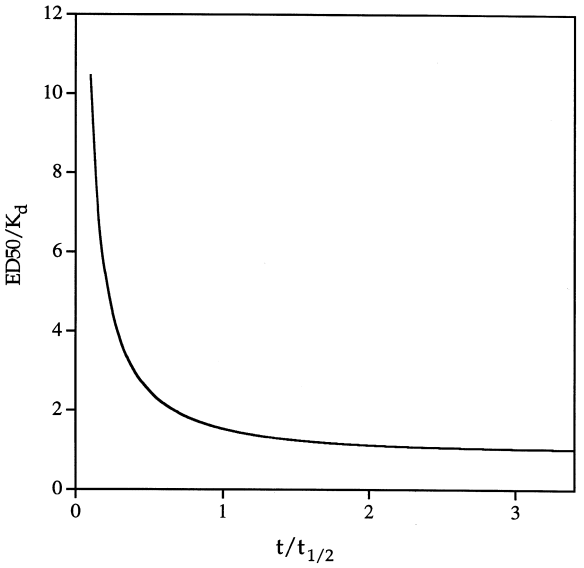

Fig. 3. Ratio of ED_{50}/K_d for a simple binding reaction $D + P = C$ as a function of reaction time $t/t_{1/2}$; peptide concentration is assumed to be constant (excess of peptide).

5.1. Examples

For biotinylated peptide used in kinetic experiments HA 307–319 we measured ED_{50} (4 h) = 170 nM and ED_{50} (24 h) = 19 nM. According to the Table 2 that gives $t_{1/2} = 230$ h ($k_2 = 0.003$ h^{-1}) and then from Table 1 we get $K_d = 1.52$ nM.

For RMBP 90–102 peptide (HFFKNIVTPR-TPA) we measured the following values of ED_{50}: ED_{50} (0.5 h) = 200 nM, ED_{50} (5 h) = 29 nM, ED_{50} (8 h) = 27 nM. This gives $t_{1/2} = 1.5$ h, that agrees with the result obtained from direct monitoring of the dissociation of peptide–DR complexes. The dissociation constant $K_d = 24$ nM, which is close to the value of ED_{50} observed at 8 h.

For the good binding peptides K_d is in the range of 1–10 nM. The on-rates of peptides most probably do not differ substantially [13]. If we have a good binding peptide with $K_d = 10$ nM and $k_1 = 2$ μM^{-1} h^{-1} (as found for the peptide used in our experimental studies), it has $t_{1/2} = \ln 2/k_2 = \ln 2/(k_1 K_d) = 35$ h. To obtain the equilibrium value of $ED_{50} = K_d$ from experimental

measurements we need to incubate the protein with peptide for more than 300 h. This is a very long experiment, and also noticeable degradation of protein might occur over a period of several days. The great advantage of the method developed above is that it allows us to obtain the dissociation constant K_d and the rate constants k_1 and k_2 in a relatively short experiment by measuring ED_{50} at several time points (for example at 4, 8 and 24 h) and processing the data as described above.

A similar method can be in general developed and applied to obtain kinetic and equilibrium characteristics for the systems described by other kinetic schemes. The practically useful application is the so-called competition assays, when two peptides (labeled and unlabeled) are involved in binding and the concentration of unlabeled peptide is varied. By comparing the ID_{50} values (concentration of unlabeled peptide at which binding of the half of the protein to the labeled peptide is inhibited) at different instants of time and having complete kinetic information (k_1 and k_2) for the labeled peptide, we can predict the off-rate constant and the equilibrium dissociation constant for the peptide studied (unlabeled peptide).

6. Conclusions

The studies performed confirmed the validity of the kinetic mechanism considered for peptide binding to MHC class II DR1DW1 molecules. The existence of the preceding step, which is the loss of endogenous peptide or some conformational change of the molecule was observed and the rate of this process was found as well as the rate constants of the subsequent binding reaction. No confirmation for the existence of the 'empty' population of DR molecules was obtained.

It was shown that ED_{50} value can be of several orders of magnitude greater than the affinity of peptide to protein, which is given by the equilibrium dissociation constant K_d of peptide–protein complex. The more strongly peptide binds to protein, the more substantial difference between ED_{50} and the affinity will be observed. Thus the best binding peptides will not be found if the search is based on the comparison of ED_{50} values obtained from the experiments performed under certain time limitations. Typically incubation period of several days may not be enough.

Using analytical features of the peptide–MHC binding model, the novel time-effective method for obtaining the equilibrium dissociation constants (affinities) and dissociation rates of peptide–protein complexes, which is based on the comparison of the experimental values of ED_{50} measured at different instants of time was developed.

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